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Molecular Immunology 44 (2007) 3173–3184
Molecular Immunology
www.elsevier.com/locate/molimm

Transcriptome analysis of human immune responses following live vaccine strain (LVS) *Francisella tularensis* vaccination

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Received 18 December 2006; received in revised form 24 January 2007; accepted 25 January 2007
Available online 8 March 2007

Abstract

The live vaccine strain (LVS) of *Francisella tularensis* is the only vaccine against tularemia available for humans, yet its mechanism of protection remains unclear. We probed human immunological responses to LVS vaccination with transcriptome analysis using PBMC samples from volunteers at time points pre- and post-vaccination. Gene modulation was highly uniform across all time points, implying commonality of vaccine responses. Principal components analysis revealed three highly distinct principal groupings: pre-vaccination (−144 h), early (+18 and +48 h), and late post-vaccination (+192 and +336 h). The most significant changes in gene expression occurred at early post-vaccination time points (≤48 h), specifically in the induction of pro-inflammatory and innate immunity-related genes. Evidence supporting modulation of innate effector function, specifically antigen processing and presentation by dendritic cells, was especially apparent. Our data indicate that the LVS strain of *F. tularensis* invokes a strong early response upon vaccination. This pattern of gene regulation may provide insightful information regarding both vaccine efficacy and immunopathogenesis that may provide insight into infection with virulent strains of *F. tularensis*. Additionally, we obtained valuable information that should prove useful in evaluation of vaccine lots as well as efficacy testing of new anti-*F. tularensis* vaccines.

Keywords: Human; Gene regulation; Vaccination; Bacterial; Molecular biology

1. Introduction

Francisella tularensis, the causative agent of tularemia, is a gram-negative, facultative, intracellular bacterium, first isolated in 1911 in association with a plague-like disease among squirrels in Tulare County, California (McCoy and Chapin, 1912; McLendon et al., 2006). *F. tularensis* is a CDC Category A threat organism due to its high infectivity rate after exposure to low numbers of organisms (10–50 bacteria), the ease of dispersal, and its potential to cause high morbidity and mortality rates among aerosol-exposed individuals (Isherwood et al., 2005; McLendon et al., 2006; Oyston and Quarry, 2005). Human tularemia presents in ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, and septic forms, and is spread through blood-feeding arthropod bites or exposure to infected vermin, soil, or water (Dennis et al., 2001). Most forms of tularemia cause mild acute symptoms of an undifferentiated febrile illness and are treatable by broad-spectrum antibiotics (Dennis et al., 2001). Primary pneumonic tularemia is rarely seen in naturally occurring cases; however, the intentional deployment of weaponized or genetically modified/antibiotic-resistant strains of *F. tularensis* presents a public health hazard estimated to result in high incapacitating casualty and mortality rates (Dennis et al., 2001). The economic impact of such an event is believed to be upwards of $6.4 billion for every 100,000 persons exposed (Kaufmann et al., 1997; Dennis et al., 2001).

The only tularemia vaccine available in the United States is an investigational new drug (IND). It is a live attenuated vaccine, comprised of the live vaccine strain (LVS) of *F. tularensis*

Abbreviations: LVS, Live vaccine strain; PCA, Principal components analysis

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doi:10.1016/j.molimm.2007.01.037

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
8 MAR 2007	**N/A**	**-**

4. TITLE AND SUBTITLE
Transcriptome analysis of human immune responses following live vaccine strain (LVS) Francisella tularensis vaccination. Molecular Immunology 44:3178-3184

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Fuller, CL Brittingham, KC Hepburn, MJ Petitt, PL Pittman, PR Bavari, S

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
United States Army Medical Research Institute of Infectious Diseases

8. PERFORMING ORGANIZATION REPORT NUMBER
TR-06-082

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)
11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT
The live vaccine strain (LVS) of Francisella tularensis is the only vaccine against tularemia available for humans, yet its mechanism of protection remains unclear. We probed human immunological responses to LVS vaccination with transcriptome analysis using PBMC samples from volunteers at time points pre- and post-vaccination. Gene modulation was highly uniform across all time points, implying commonality of vaccine responses. Principal components analysis revealed three highly distinct principal groupings: pre-vaccination (-144h), early (+18 and +48h), and late post-vaccination (+192 and +336h). The most significant changes in gene expression occurred at early post-vaccination time points (

15. SUBJECT TERMS
Franciscella tularensis, tularemia, gene regulation, transcriptome analysis, microarray, Human studies, volunteers

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**12**	

(Eigelsbach and Downs, 1961). The vaccine is administered by scarification of the volar surface of the forearm and creates a persistent papular/pustular lesion at the inoculation site similar to infection with virulent *F. tularensis* infection (Burke, 1977). The vaccine, in use for 50 years, has substantially lowered the number of laboratory-acquired incidents of tularemia (Oyston and Quarry, 2005; Isherwood et al., 2005; Burke, 1977).

Relatively little is understood regarding protection against tularemia in humans. Much of the data regarding *F. tularensis* immunopathogenesis, as well as the mechanism of protection afforded by the vaccine, comes from murine models. Humoral immunity was previously believed to be important as passive protection against LVS challenge was demonstrated in mice given immune serum from LVS-vaccinated humans (Drabick et al., 1994). However, vaccine-induced humoral responses to LVS may play no role in protection against human pathogenic *F. tularensis* strains (Tarnvik, 1989). Instead, as with most intracellular pathogens, cell-mediated responses are thought to be critical in long-lasting protective immunity (Waag et al., 1992, 1996; Tarnvik et al., 1985; Surcel et al., 1991; Sjostedt et al., 1992). Protection of mice from lethal challenge with LVS develops as early as 2–3 days after intradermal vaccination (Elkins et al., 1992). Similarly, intranasal infection of mice with lethal doses of LVS results in rapid NK cell recruitment and activation in the lungs (Lopez et al., 2004). Recent studies in our laboratory suggest that early innate responses (<48 h) occur in humans after LVS vaccination and may correlate with protective immunity (Fuller et al., 2006). Rapid development of immunity after vaccination in mice also suggests that protective mechanisms are partly attributable to strong initial innate immune responses. The cellular components of these early immune responses, such as NK cells, neutrophils, and DCs, have only recently become the focus of attention in murine and human studies of LVS vaccination (Telepnev et al., 2005; Sjostedt et al., 1994; Malik et al., 2006; Isherwood et al., 2005; Fuller et al., 2006; Elkins et al., 2003; Cole et al., 2006; Barker et al., 2006).

While a FDA-approved vaccine for *F. tularensis* may be on the horizon, the methods used to determine the mechanism of protective immunity have not been re-visited in decades. Here, we examined PBMC samples from vaccinees at varying time points in an effort to delineate early immune responses upon which long lasting protection is built. Using modern transcriptome analysis following vaccination, we gained a better understanding of the immunology of LVS vaccination and perhaps the responses necessary for long-lasting immunity. These data will aid in evaluating host response to virulent *F. tularensis* and because LVS is a uniquely live vaccine, the data offer a seldom-observed study of human response to bacterial infection.

2. Materials and methods

2.1. Collection and preparation of PBMCs

Volunteers were recruited from U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) personnel at risk of laboratory exposure to *F. tularensis*. A human use clinical protocol to collect peripheral blood samples was approved by institutional review boards at USAMRIID (Human Use Committee FY04-16). Donors provided informed consent and met eligibility criteria. Six healthy adults (three males and two females, 22–54 years of age) received a primary LVS vaccination (*F. tularensis* vaccine, NDBR 101, Lot 4) and donated peripheral blood, 6 days prior to vaccination, 18 h after vaccination, 48 h after vaccination, 8 days after vaccination, and 14 days after vaccination. Inoculum consisted of a single 0.6-ml drop of LVS vaccine (2×10^9 colony-forming units (cfu) per ml) delivered intradermally by bifurcated needle. All vaccinees had positive "takes" (initial formation of small papule which then ulcerates). Vaccinations were deemed clinically successful by microagglutination assays (titers ranged from 1:20 to 1:1280) at day +28(Massey and Mangiafico, 1974). Blood was collected into heparinized syringes under sterile conditions at the medical clinic and transferred to a biosafety level (BSL)-2 laboratory for processing. PBMCs were purified from whole blood by Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ, USA) density gradient centrifugation.

2.2. RNA isolation

The cell pellet was resuspended in 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA) solution and pipetted multiple times to dissolve all clumps and break up the cellular DNA. An additional 2 ml of TRIzol was added to the cell lysate and mixed well. One milliliter of TRIzol-lysed solutions were then was dispensed into 1.5 ml Eppendorf centrifuge tubes and snap frozen in a bath of 95% ethanol and dry ice, then transferred to −70 °C until RNA was purified. Total cellular RNA was isolated according to the manufacturer's specifications followed by purification with an affinity resin column (Qiagen, Inc., Valencia, CA, USA). The quality and concentration of the RNA were determined by measuring the absorbance at 260 and 280 nm. RNA integrity was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All plastic materials used in the lysing procedure were certified RNAse- and DNAse-free and all samples were handled with latex glove-covered hands in a BSL-2 laminar flow cabinet to minimize deposition of environmental RNAse onto or into sample tubes.

2.3. Transcriptome/microarray analysis

RNA samples used in this study were checked for evidence of degradation and integrity and had a minimum A260/A280 ratio of > 1.9 and a minimum 28S/18S ratio of > 1.6 (2100-Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA). The Human Genome U133+2 array, which consists of 54,654 probe sets representing approximately 33,000 human genes, was used (Affymetrix, Inc., Santa Clara, CA, USA). GeneChip analysis was performed with Microarray Analysis Suite (MAS) 5.0, Data Mining Tool (DMT) 2.0, and Microarray Database software (Affymetrix, Inc., Santa Clara, CA, USA). All of the probe sets represented on the GeneChip arrays were globally normalized and scaled to a signal intensity of 100.

Various microarray RNA integrity indicators were used to filter samples for quality in the final analysis. Principal component analysis (PCA) was used to quickly identify outlier arrays. Microarray quality control included the following manufacturer's standard parameters: noise (RawQ), consistent number of genes detected as present across arrays, consistent scale factors, and consistent beta-actin and GAPDH 5′/3′ signal ratios (Affymetrix, 2000). Only those samples that passed Gene Logic, Inc.'s quality control metrics for each of these parameters were added to its Gene Enterprise System® Software for storage and analysis. Gene expression values were floored to 1 and then $\log_2$-transformed. PCA and analysis of the global correlation matrix were performed based on all probe sets. The covariance PCA and correlation-based analyses were done within Partek® software (Partek, Inc., St. Louis, MO, USA). *T*-tests were performed for each probe set to identify statistically significant changes in gene expression between pre-vaccination and each of the post-vaccination groups. A gene had to be $p \leq 0.01$ with a fold change of at least 2 in either direction to be considered significantly regulated for individual pair-wise comparisons. Note that the pair-wise comparisons were primarily used to establish relative numbers of genes regulated across time such that adjustment of *p*-values was not necessary. Potential markers of vaccination were selected statistically using the ANOVA-based pattern-matching techniques described below.

2.4. Gene filtering, pattern matching, and clustering

Five primary patterns of regulation of interest were investigated based on principal trends within the data set. To determine genes that robustly correlated with these five patterns, a three-step process was implemented. First, a one-way ANOVA was performed to calculate *F*-scores across the five groups of samples. An *F*-score of 10, which translates to a $p < 0.001$ based on random sampling distributions, was chosen as a cut-off for inclusion such that a score above 10 was deemed evidence that a regulation event had occurred for one or more of the five groups. Individual gene scatter, directly visualized according to group, was used to validate this *F*-score as robust. Second, the absolute value of the Pearson's correlation was calculated for each probe set relative to the five predetermined patterns of regulation. A cut-off of 0.95 was chosen to indicate highly correlated probe sets. We visually inspected individual probe sets to validate this cut-off. Finally, we conducted a *k*-means Euclidean distance clustering of the mean signal intensity for probe sets that passed each of the previous criteria using $k = 8$, 10, 12, and 14 clusters after global mean normalization. $k = 10$ was chosen as optimal due to generation of clusters by up- or down-regulation events and by separation of probe sets into clusters of high degrees of regulation and those of lesser regulation. The distinction of high versus low degrees of regulation was made as markers of a high degree of regulation would be more likely to validate on another platform than those of lesser regulation. All clustering was performed in Spotfire® DecisionSite™ 8.0 (SpotFire, Inc., Somerville, MA, USA) and ArrayMiner 5.3.2 (SOM; Optimal Design, Brussels, Belgium). Gene annotations utilized Gene (http://www.ncbi.nlm.nih.gov/entrez/) and Bioinformatic Harvester databases (Liebel et al., 2004, 2005).

3. Results

3.1. Response to LVS vaccination is highly comparable from person-to-person

We designed our study to allow a kinetic assessment of global gene regulation upon vaccination with LVS. Five time points were chosen representing pre-vaccination (−144 h) as well as early (+18 and +48 h) and late (+192 and +336 h) times post-vaccination (Fig. 1A). These time points allowed assessment of gene regulation associated with innate (early) and acquired (late) immune responses. The formation of pustules at the inoculation site is accepted as anecdotal evidence of a positive "take" for LVS vaccination and is visual proof of rapid innate immune response to the live bacterial vaccine (Fig. 1B; +18 h). Rapid induction of this inflammatory response was visible as early as +0.5 h and was clearly evident at +18 h with all volunteers developing pustules at the inoculation site (Fig. 1B). Pustules were resolved by +672 h when all volunteers

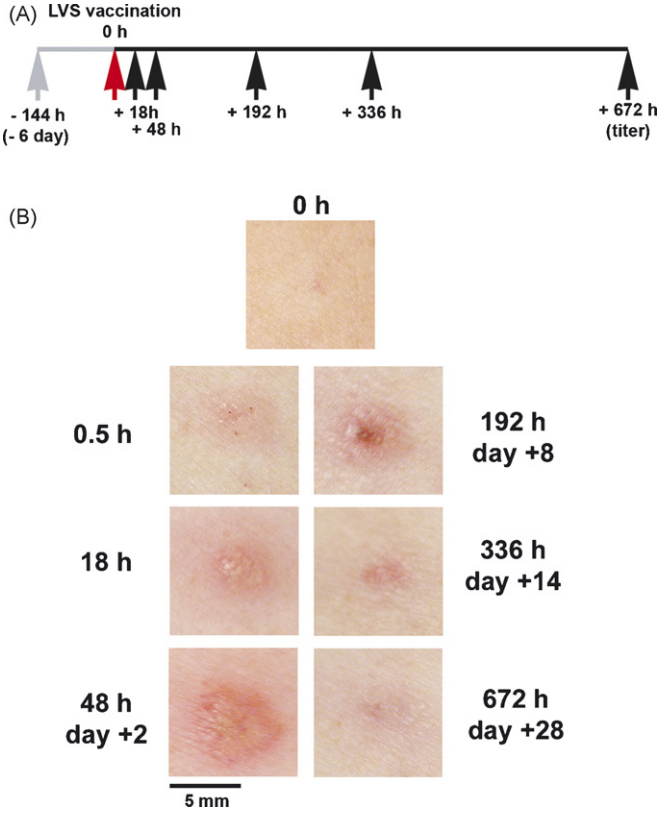

Fig. 1. Study design yielded data confirming commonality of LVS vaccine response. (A) Five consenting volunteers donated PBMCs 6 days before LVS vaccination, then 18, 48, 192, and 336 h post-vaccination. PBMCs were processed for RNA isolation as described in the Section 2. (B) Photographic documentation of vaccine site pustule formation and ulceration of the volar surface of the arm at the indicated time points, post-vaccination. Photographs are representative of reactions seen in all volunteers.

Table 1
Antibody titers volunteers developed *F. tularensis*-specific antibodies following vaccination

Volunteer	Pre-vaccination titer	Post-vaccination titer
22	0	160
23	0	20
24	0	320
27	0	40
32	0	1280

developed positive anti-*F. tularensis* titers (mean 1:364 ± 526; Table 1).

Correlation mapping of all patients, at all time points, using all 54,000+ probe sets (i.e., gene fragments) was performed to measure the overall similarity of samples to one another. Visualization by heatmap revealed that no individual sample-to-sample comparison had less than a 0.89 correlation and that average correlations within each group were above 0.95 (Fig. 2, enlarged in Supplemental Fig. 1). This indicated high correlation across all samples and that no strong outlier behavior was present on a global gene expression basis. These data signify commonality in human responses to LVS vaccination and lend weight to assertions that the results are applicable to a broad range of vaccinees and their predicted response to the vaccine.

3.2. Global expression pattern analysis yields three principal groupings

To determine the relationship between global expression for each patient (Fig. 2) and various time points, principal component analysis was performed using all probe sets on the array (Fig. 3A, enlarged in Supplemental Fig. 2). Volunteers with similar responses at a given time point appeared close to each other on the PCA plot and the converse was also true. The first principal component (*x*-axis) resulted in an inferred biological component of variability between the early post-vaccination response and the rest of the experiment, with 11% of total experimental variability explained. The second principal component (*y*-axis) resulted in an inferred biological component of variability between three principal groupings: pre-vaccine (−144 h), early post-vaccination (+18 and +48 h), and late post-vaccination (+192 and +336 h). The chart also reveals an apparent outlier, 22a, the pre-vaccination (−144 h) time point for volunteer 22. Further statistical analysis argued that 22a was not a severe outlier and internal controls did not suggest technical errors. Follow-up with this volunteer revealed the volunteer had received another vaccination fewer than 28 days before the pre-vaccination time point. Titer results from volunteer 22 at +672 h (day 28) failed to reveal any gross differences in this individual's response to vaccination. In fact, volunteer 22 had the median titer for all five volunteers (Table 1). While this biological variability was noted, volunteer 22 was found to have responses similar to other volunteers at all other time points and was not removed from downstream analysis.

While it is true that proteins with stable message are certainly involved in immune responses to vaccination, the impact of genes that show no change are difficult to assess. Therefore, we sought to examine the genes that showed the most change from pre-vaccination to post-vaccination, and from time point to time point. Fig. 3B represents the relative number of genes that changed greater than twofold (up, down, and up/down

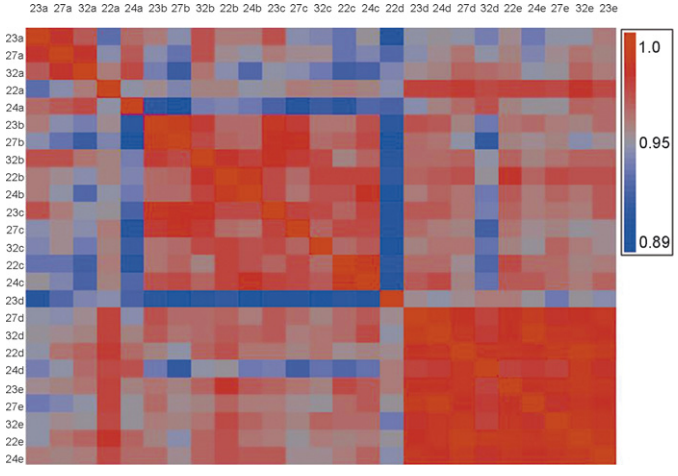

Fig. 2. Heatmap visualization of correlation matrix for all five volunteers (22, 23, 24, 27, and 32) at all five time points (a, −6 d; b, 18 h; c, 48 h; d, 192 h; e, 336 h) all using 54000+ probe sets. The plot indicated high correlation across all samples and that no sample-to-sample comparison showed less than a 0.89 correlation. Further, no sample showed an average correlation to all other samples of less than 0.92.

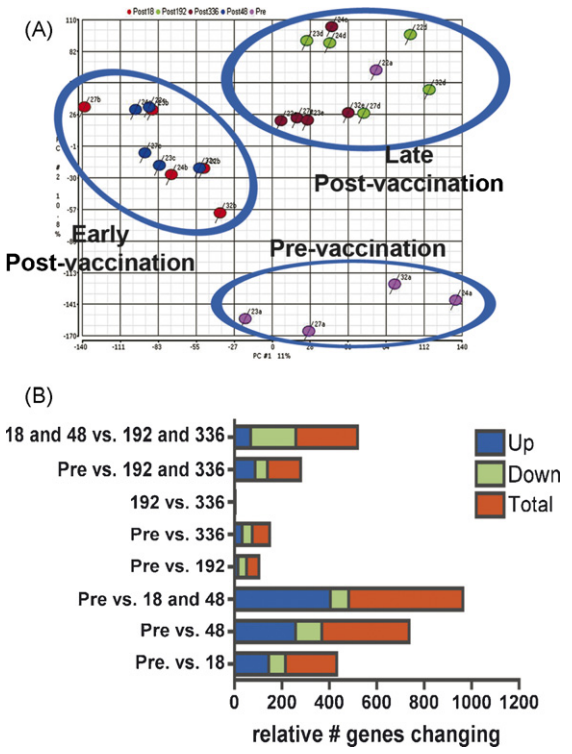

Fig. 3. Covariance principal component analysis and fold change assessment. (A) PCA analysis revealed the lack of severe outliers and the presence of three principal groupings (indicated by circles): pre-vaccination, −144 h; early post-vaccination, 18 and 48 h; and late post-vaccination, 192 and 336 h. (B) Pairwise comparison of number of genes changing more than twofold (up or down) relative to the earlier time point. A paired Student's *t*-test indicated $p < 0.01$ for all comparisons.

combined – total) from one time point to another. The highest number of changes came between pre-vaccination versus 48 h and pre- versus 18 h post-vaccination, the majority of which were up-regulation events. The least number of changes were observed for 192 h versus 336 h, then pre- versus 192 h, and pre- versus 336 h. These observations point to the very earliest time points being the source of the largest gene modulation events and later events having the very least amount of modulation. In further evaluation of the principal groupings resulting from PCA, assessments between each of the three groups, pre-, early post-, and late post-vaccination were performed. There were three to four times as many genes changing in early post-vaccination versus pre-vaccination than in late post-vaccination versus pre-vaccination groups. Also, genes were down-regulated three times more in early post-vaccination versus late post-vaccination. This analysis quantifies data from the PCA plot (Fig. 3A) and stated that: (1) there were, in fact, three intra-related groups; (2) that the largest difference between any of the groups was between pre-vaccination and early post-vaccination; and (3) late post-vaccination was more similar to pre-vaccination than to early post-vaccination. Together, these data corroborate previous studies from our laboratory and others suggesting that the earliest responses to vaccination or infection may play key roles in anti-*F. tularensis* immune responses (Telepnev et al., 2005; Malik et al., 2006; Katz et al., 2006; Isherwood et al., 2005; Fuller et al., 2006; Elkins et al., 2003).

3.3. Gene expression patterns reveal early peak in LVS-induced gene modulation

ANOVA-based contrast analysis allowed us to categorize the kinetics of gene modulation during LVS vaccination and search for genes modulated in a defined manner. The 10 most prevalent patterns are depicted in Fig. 4A. Mean normalized signal intensity for all five volunteers was plotted (positive or negative) versus the five time points assessed, with individual lines representing individual genes meeting the statistical cut-off to be included in each *k*-means cluster group (Fig. 4B). Despite having relatively high numbers of genes fitting their pattern and meeting the statistical standards set, clusters 1 and 2 were noisy and showed genes without large responses relative to pre-vaccination. In contrast, clusters 3–10 had large relative responses and cluster variability was small. The four most prevalent patterns in order were: "down early" (pattern 4), "up early" (pattern 6), "sustained up" (pattern 3), and "up late" (pattern 5). Gene modulation patterns were further analyzed using self-organizing map (SOM) in which the data were fit to data-defined patterns instead of predetermined patterns. The six SOMs that best fit the data were similar to patterns 3–6, 8, and 9, with the relative number of genes in each group matching those depicted in Fig. 4. These *k*-means clustering patterns mirrored the fold-change and PCA analysis (Fig. 3) in describing the largest differences in gene modulation at 48 h or less (early pre-vaccination). Changes primarily reflect down- (down early) and up- (up early) regulation of genes at ≤48 h and subsequent return to pre-vaccination levels at days 8–14. The pattern analysis expounds on the importance of early events by suggesting that

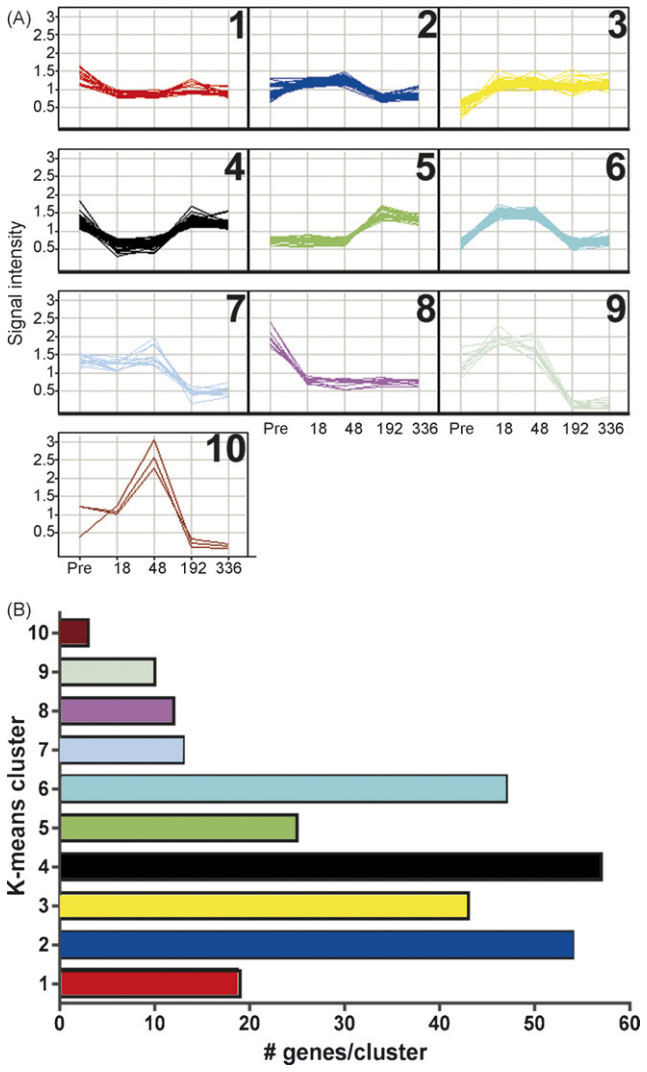

Fig. 4. *k*-means clustering of genes reveals 10 prevalent patterns. (A) Mean normalized signal intensities were examined for changes in regulation across all five time points. ANOVA-based pattern-matching analysis revealed the 10 most highly observed kinetic patterns. Lines represent individual gene patterns allowing assessment of "noise" in each group. Patterns with *F*-scores > 10 were considered significant. (B) Quantification of genes that made the statistical cut-off (Pearson's correlation of ± 0.95) to be included in a given cluster group represented in 4A. The two combined statistical filters yielded tightly observable clusters of gene regulation across time.

of the responses to LVS vaccination, the most ordered and most prevalent are those that occurred at early times post-vaccination.

3.4. Immune response genes reflect predominance of early modulation events

The high degree of kinetic similarity among these responding genes suggests activation of a distinct orchestrated biological response to stimuli, in this case, LVS vaccination. Further examination of the biological function of genes in each of the four most prevalent patterns yielded genes involved in six general types of cellular processes: immune-related, cell cycle-related, apoptosis-related, biosynthesis/metabolism, other/ambiguous (genes that are nearly ubiquitous in several processes, e.g.

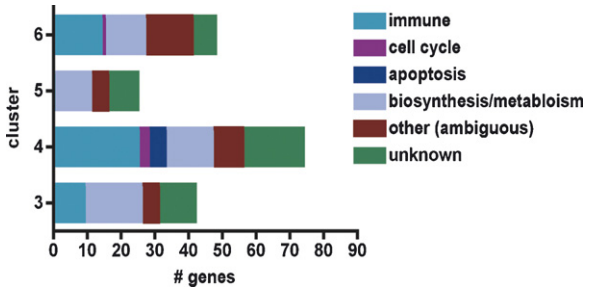

Fig. 5. Categorization of clustered genes reveals strong bias towards immune and biosynthesis/metabolism-related genes. Genes clustered in the four most prevalent patterns were sorted by function. Genes were categorized based on relatedness to host defense (immune), cell cycle regulation and proliferation, cell death and apoptosis, general protein/lipid/carbohydrate synthesis or metabolism, unknown function, or function that were common to too many pathways to allow obvious categorization. Not that some genes fell into more than one category.

MAPK), and those of unknown function (Fig. 5). The most frequently observed pattern, pattern 4 or "down early", was also the pattern that contained the highest number of immune-related genes. The inverse pattern, pattern 6 or "up early", also showed a significant bias towards immunity-related gene modulation. The two patterns suggest that a great number of genes are selectively up- or down-regulated in a timeframe more consistent with innate effector activation than acquired immune effector activation. In fact, only pattern 5, "up late," showed genetic modulation consistent with acquired immune effector activation. Despite its kinetics, pattern 5 had the fewest matching immune-related genes of all patterns examined.

A better indication of the immune events occurring as a consequence of LVS vaccination was obtained by examining specific immune-related genes modulated in each pattern (Tables 2–5). Of the 42 genes that fit pattern 3 "sustained up," approximately nine could be linked directly to immune function (Table 2). Among these were innate immune system defense mechanisms such as regulators of endocytosis/phagocytosis, granule exocytosis, chemotaxis, and inflammatory cytokines. These genes, induced upon vaccination, remained elevated throughout the 336 h of the study, suggesting a continual need for effector mechanisms controlled by these genes.

Pattern 4, "down early," represents genes that were down-regulated in the early timeframe associated with initiation of immune responses. This group had the largest number of genes matching its pattern. Out of those 56 matches, 21 genes had immune-related functions (Table 3). Among these were regulators of chemotaxis/adhesion, T-cell signal transduction, cytoskeletal remodeling, proliferation, and pro-inflammatory cytokine response. Several immune-related genes were just below our stringent threshold (see Section 2) for inclusion in pattern 4 "down innate" but worthy of note. Included in this group were: *IFNA10/IFNA17, CD28, CD3E, CD3ζ, CD3G, CD40, FASLG* (CD178), killer cell lectin-like receptor *(KLR)-B1, KLRC3*, natural killer cell group *(NKG)-7*, natural killer-tumor recognition sequence *(NKTR), STAT3*, TNF receptor-associated factor *(TRAF)-1,* and *TRAF5*. These genes are intimately associated with pro-inflammatory immune response by NK cells, NKT cells, and TLR-bearing innate immune effector cells such as DCs, monocytes, and neutrophils.

Down-regulation of the genes listed in Table 3 suggests an attempt to modify the environment in which immune system activation will take place by dictating the recruitment of cells, the way cells receive stimulatory signals, and the cytokine milieu in which cells receive this signal. Down-regulation of these genes could suggest negative regulation of pro-inflammatory immune responses at a very early stage. This seems unlikely given the visible immune reaction at the site of vaccination (Fig. 1B). Conversely, down-regulation may indicate genes that have already been triggered and are now being dampened to prevent an overzealous inflammatory response. It is likely that the protein products of these genes were being produced at +18 to 48 h and thus the transcriptional attenuation observed indicates preparation for subsequent down-regulation. Previous reports of

Table 2
Fuller et al Pattern 3 "sustained up" genes involved in immune function

Gene symbol	Gene name	Biological process	Gene ID
LRP1	Low-density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)	Cell proliferation, endo/phagocytosis of apoptotic cells, lipid metabolism, cell adhesion, interacts with NOS1AP, C3, furin, lactotransferrin	4035
M6PR	Mannose-6-phosphate receptor (cation dependent)	Transport of phosphorylated lysosomal enzymes from the golgi complex and the cell surface to lysosomes, receptor-mediated endocytosis	4074
TXNIP	Thioredoxin interacting protein	Novel regulators of tumor necrosis factor signaling and inflammation in endothelial cells	10628
VAMP8	Vesicle-associated membrane protein 8 (endobrevin)	Required for platelet granule secretion	8673
MAPKAPK3	Mitogen-activated protein kinase-activated protein kinase 3	Response to stress, signal transduction, associated with IL1alpha and beta regulation	7867
SPN	Sialophorin (gpL115, leukosialin, CD43)	Cellular defense response, chemotaxis, associated with CD44, FYN, WASP, P-selectin, ICAM2	6693
CMRF-35H/Irp60	Leukocyte membrane antigen (CD300a)	Inhibitor of IgE-induced mast cell degranulation, NK cell cytolysis inhibitor	11314
ARRB1	Arrestin, beta 1	Sensory perception, signal transduction, regulating receptor-mediated immune functions	408
DKFZP564J0863	DKFZP564J0863 protein/ATL3	Immune response	25923

Table 3
Pattern 4 "down early" regulated genes involved in immunity

Gene Symbol	Gene Name	Biological Process	Gene ID
CD96	CD96 antigen (TACTILE)	Cell adhesion, immune response (NK and T-cells), antigen presentation	10225
NAP1L1	Nucleosome assembly protein 1-like 1	DNA replication, positive regulation of cell proliferation	4673
RORA	RAR-related orphan receptor A	Regulation of transcription, signal transduction, reduces VCAM-1/ICAM-1 expression	6095
CCL5	Chemokine (C-C motif) ligand 5 (RANTES)	Cell adhesion, cellular defense response, chemotaxis, exocytosis, inflammatory response, response to oxidative stress, induced by TNF alpha and IL1alpha	574178
PTPRCAP	Protein tyrosine phosphatase, receptor type, C-associated protein (CD45-associated protein)	Defense response, lymphocyte signaling	5790
TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	Apoptosis, signal transduction, lymphocyte proliferation	8718
TRBV19	T-cell receptor beta variable 19	T-cell receptor, signal transduction, activation	28568
CD3D	CD3D antigen, delta polypeptide (TiT3 complex)	T-cell receptor complex, signal transduction, activation	915
ZAP70	Zeta-chain (TCR) associated protein kinase 70 kDa	Protein amino acid phosphorylation, lymphocyte protein kinase cascade	7535
NUMA1	Nuclear mitotic apparatus protein 1	Mitotic anaphase, cytoskeletal rearrangement, caspase activation	4926
PRKCH	Protein kinase C, eta	Intracellular signaling cascade (Akt and mTOR)	5583
KLF2	Kruppel-like factor 2	Negative regulation of gamma globulin genes, induction of eNOS, apoptosis, IL-2 promoter activation	10365
NACA	Nascent-polypeptide-associated complex alpha polypeptide	Protein biosynthesis, co-activator of Jun transcription factors and FADD signaling	4666
STMN3	Stathmin-like 3	Intracellular signaling cascade, microtubule-destabilizing phosphoproteins	50861
GSPT1	G1 to S phase transition 1	G1/S transition of mitotic cell cycle, mRNA catabolism, JNK1 interaction, apoptosis block	2950
F11R	F11 receptor/junction adhesion molecule (JAM1)/CD321	Cell motility, inflammatory response, NFkappaB activation, negative regulation by TNF alpha and IFN gamma, associated with CD3D	50848
PAR1/UBE3A	Prader–Willi syndrome chromosome region 1, GMCSFRalpha precursor, IL3Ralpha precursor (CD123)	Brain development, proteolysis and peptidolysis, regulation of transcription, DNA-dependent, ubiquitin cycle, ubiquitin-dependent protein catabolism, associates with Cbl and BLK	7337
RHOH	ras homolog gene family, member H	T-cell differentiation, negative regulation of I-kappaB kinase/NF-kappaB cascade, regulation of transcription	399
PPP2R5C	Protein phosphatase 2, regulatory subunit B (B56), gamma isoform	Signal transduction, paxillin and PKC delta-associated	5527
GA17/PCID1/ hfl-B5	Dendritic cell protein/PCI domain containing 1	Herpesvirus entry mediator	10480

innate immune effector cell activation at these time points support this hypothesis (Katz et al., 2006; Fuller et al., 2006; Elkins et al., 1992; Elkins et al., 2003).

Despite kinetics suggesting the specific up-regulation of genes during acquired immune system activation, pattern 5 "up late" had the least immune-related gene modulation (Table 4). Only the lymphocyte cell-specific tyrosine kinase *LCK* was up-regulated in a manner consistent with pattern 5. LCK plays a vital role in surface receptor signaling in NK, NKT, and T-cells. Because transcriptional analysis was performed on total PBMC populations, it is difficult to interpret up-regulation of a kinase as ubiquitous as LCK. The timeframe in which *LCK* was activated does imply preparation for an immune response. The predominance of biosynthetic and metabolic genes matching this pattern (Fig. 5) was consistent with up-regulation of a coordinated immune response, whether this was in the form of a secondary innate response or an adaptive response was unclear.

Pattern 6 or "up early" represents genes that are turned on in a timeframe consistent with activation of innate effectors such as mast cells, DCs, neutrophils, macrophages, granulocytes, and monocytes. The immune-related genes in pattern 6 are highly consistent with activation of these innate immune system cells (Table 5). Multiple indicators of innate functional response such as phagocytosis, exocytosis, super oxide formation, antigen processing, cytokine/chemokine production, and signal transduction are among the up-regulated genes. Nearly every up-regulated gene listed can be directly linked to a specific innate immune system cell and an associated effector function.

Table 4
Pattern 5 "up late" regulated genes involved in immunity

Gene symbol	Gene name	Biological process	Gene ID
LCK	Lymphocyte cell-specific protein tyrosine kinase	Intracellular signaling cascade, protein phosphorylation, associated with CBL, CTLA4, ZAP70, CD4, CBL, JAK3, CD2, SYK, CD8, CD45	3932

Table 5
Pattern 6 "up early" regulated genes involved in immunity

Gene Symbol	Gene Name	Biological Process	Gene ID
CREG1	Cellular repressor of E1A-stimulated genes 1	Antagonizes transcriptional activation and cellular transformation by E1A	8804
LAMP2	Lysosomal-associated membrane protein 2 (CD107b)	Protection, maintenance, and adhesion of the lysosome, promoting immunological recognition and antigen presentation, cytolytic granule exocytosis marker, cathepsin, CD63 associated	3920
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor for (CD32)	Immune response, lymphocyte activation: CRP, BLK, LAT, HCK, PI3K, SHC1	2212
DCL-1	Type I transmembrane C-type lectin receptor DCL-1 (CD302)- DEC205 ligand	Mono/mac and DC (expressed on migrating DC) receptor, associated with CD8, CD40L, CD80, CD86, NKp30 (CD337), IL4, IL-12Ralpha, IL15, GMCSF	9936
GLIPR1	GLI pathogenesis-related 1 (glioma)	CD58 (LFA3) and CD2 associated, costimulation	11010
CSF2RB	Colony-stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage) – IL3/IL5 receptor low-affinity	Antimicrobial humoral response, cytokine and chemokine-mediated signaling pathway: JAK2, signal transduction: LYN SHC1, PKC beta	1439
QPCT	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	Protein modification, proteolysis, MICA and HLA-B associated	25797
RNASE2	Ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin) EDN/RNS2	RNA catabolism, selectively chemotactic for dendritic cells, pro-inflammatory DCs, neutrophils, and granulocytes	6036
IL18	Interleukin 18 (interferon-gamma-inducing factor)	Pro-inflammatory immune response, chemokine production, apoptosis, cytokine production: GMCSF, IFN-gamma, TNF, IL1alpha, IL2 IL13, IL2, positive regulation of activated T-cell proliferation, adhesion: ICAM1, VCAM1	3606
CD36	CD36 antigen (collagen type I receptor, thrombospondin receptor)	Blood coagulation, cell adhesion ICAM1, phagocytosis, CD9-assoiated, LYN signaling	948
LY96/MD-2	Lymphocyte antigen 96/ TLR4 associated	Antibacterial humoral response, TLR4 (LPS) linked signal transduction: MYD88, IRAK1, TLR2, TLR9, TLR10,CD14, PI3K, NFkappaB, cellular defense response, inflammatory response: IL8, TNF	23643
LILRA6 (ILT8/LILRB6)	(Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2, leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3, leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 6)	Inhibitory immunoreceptors expressed on monocytes, B cells, dendritic cells and natural killer (NK) cells, associated with TLR2, DC-SIGN, IL4, SLAMF1, KLRA1, ITLN1	79168
CD39/ ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1 (lymphoid cell activation antigen)	Antimicrobial humoral response, blood coagulation, cell adhesion, cell–cell signaling, expressed primarily on activated lymphoid cells, marks differentiation of granulocytic and monocytic cells	953
NCF2	Neutrophil cytosolic factor 2	Cellular defense response, superoxide metabolism	4688
CD116/CSF2RA	Colony-stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)	Controls the production, differentiation, and function of granulocytes and macrophages, associated with CXCR3, JAK2, c-fos, gamma IP10, c-Kit, IL3R	1438
IL13RA1	Interleukin 13 receptor, alpha 1	Binds IL13 with a low affinity, alternate accessory protein common cytokine receptor gamma chain for IL4 signaling, associated with TYK2, JAK1, STAT3, STAT6, IFN gamma, IL5, IL7, IL13, IL19	3597
SCAP2	src family-associated phosphoprotein 2	Protein complex assembly, signal transduction, associated with FYB, FYN, HCK, LYN, PTPRH and PRAM1	8935
IRAK3	Interleukin-1 receptor-associated kinase 3	Cytokine and chemokine-mediated signaling pathway, inhibits dissociation of IRAK1 and IRAK4 from the toll- like receptor signaling complex by either inhibiting the phosphorylation of IRAK1 and IRAK4 or stabilizing the receptor complex	11213
CLEC4A/CLECSF6	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 6	Antimicrobial humoral response, cell adhesion, cell surface receptor linked signal transduction, inflammatory immune response, linked to CD99 and other CTL/CTLD superfamily members in the natural killer gene complex region	50856

Genes worthy of note that matched pattern 6 but did not meet cut-off criteria were *CASP4*, *FAS (CD95)*, *TANK*, and *CR1*, all of which are associated with cytolysis and recognition of pathogens or infected cells. These data are highly consistent with previous work from our laboratory and others, which noted strong innate immune system responses immediately following LVS vaccination or challenge (Telepnev et al., 2005; Isherwood et al., 2005; Fuller et al., 2006; Elkins et al., 2003).

3.5. Genes regulating DC function are strongly activated by LVS vaccination

The substantial decrease in laboratory-acquired tularemia in conjunction with knowledge gained from murine tularemia models suggests that the LVS vaccine is capable of inducing a strong cell-mediated response (Tarnvik et al., 1985; Tarnvik, 1989; Isherwood et al., 2005; Fuller et al., 2006; Elkins et al., 1992, 2003; Dennis et al., 2001). While the innate immune system may be important for initial response to *F. tularensis* infection, its role in generating long-lasting protective immunity mediated by the adaptive immune system is less clear. The keystone event in linking strong innate responses to well-primed adaptive responses is activation of DCs (Mocikat et al., 2003; Katz et al., 2006; Fuller et al., 2006). Closer examination of genes associated with the professional antigen processing and presentation duties of DC and other non-professional antigen presenting cells reveals a strong tendency towards up-regulation at early time points followed either by down-regulation or sustained high levels of gene expression (Fig. 6).

Genes involved in antigen processing and presentation such as *DC-SIGN* (CD209), MHC class I (β*2m and TAP1*), MHC Class II, and CD1 all appear to be up-regulated during the first 48 h post-vaccination. This indicated that DCs and other antigen-presenting cells were recognizing components of the LVS vaccine in a manner sufficient to induce up-regulation of presentation molecules for naïve αβ and γδ T-cells. The quality of the DC-T cell activation event was largely dependent on costimulatory and adhesion molecules present on the DC during activation. Costimulatory molecules *B7-1* and *B7-2* (CD80 and CD86) were up-regulated at less than 48 h. CD80 was considerably down-regulated at 192–336 h while CD86 expression was lower at 192 h but then increased at 336 h. *ICOS*, an important positive regulator of anti-inflammatory lymphocytes was down-regulated throughout the time course. Up-regulation of ICAM-1, the ligand for lymphocyte integrin CD11/18, is associated with induction by pro-inflammatory cytokines like *IFNγ*, *TNFα*, *IL-1α*, and *IL-18*, was noted only at 48 h post-vaccination and was notably down-regulated at all other time points. *ICAM2*, the ligand for lymphocyte LFA-1 associated with NK cell-mediated clearance and lymphocyte recruitment, was up-regulated throughout the time course. CD44 and CD58, both known to mediate lymphocyte activation and homing as a result of up-regulation by pro-inflammatory cytokines, are also adhesion molecules which were specifically up-regulated 18–48 h post-vaccination. For a number of costimulatory/adhesion genes, their ligand/functional counterparts were also up-regulated in a complementary fashion (e.g., *CD58/GLIPR*, *LILRA6/DC-SIGN*, *ICAM1/CD36*, *CD44/SPN*) (Tables 2–5) again suggesting a finely orchestrated response to LVS vaccination. Recent reports from murine model systems also underscore the importance of DC processing and presentation effector functions in initiation (<20 h) of anti-*F. tularensis* responses (Katz et al., 2006).

Data suggesting that DC and other innate immune system cells are strongly activated early begs the question of how cells are recognizing LVS components. The greatest surprise in our analysis was the lack of *NOD1/2* (*CARD4* and *CARD15*) regulation in response to vaccination (Fig. 7). *F. tularensis* is an intracellular bacterium and therefore pattern recognition motifs that specifically sample the intracellular environment seem likely to be involved in recognition. Yet NOD1/2 signaling did not seem to modulate with respect to vaccination. The data were unable to rule out use of NOD1/2 as recognition receptors for LVS as no measure of protein level was obtained. Murine studies have implied the importance of TLR2- and 6-mediated recognition of *F. tularensis* by DCs (Katz et al., 2006). In our study, all TLRs were up-regulated across all

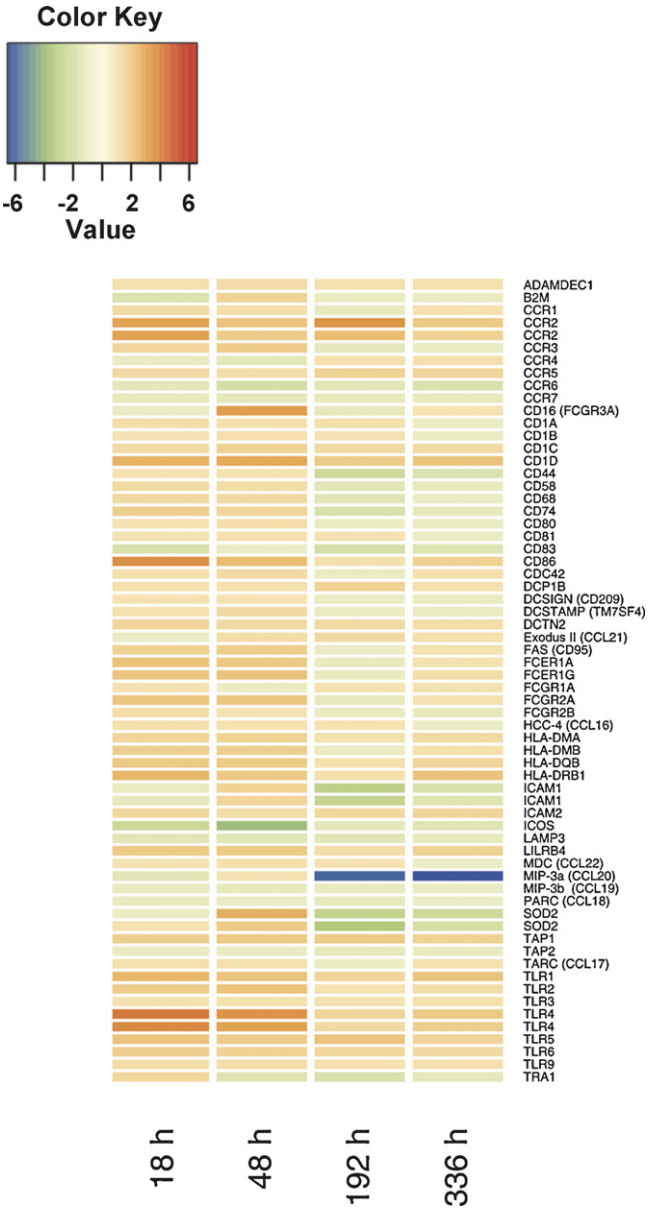

Fig. 6. Heatmap visualization of DC and antigen-presenting associated gene modulation was either up- or down-regulated at early times post-vaccination. The heatmap represents mean fold-change differences in intensity as compared to the pre-vaccination group for five donors at four post-vaccination time points for the indicated antigen presenting- and processing-associated genes. Multiple listings of a single gene indicate that gene is represented by more than one gene fragment in the array.

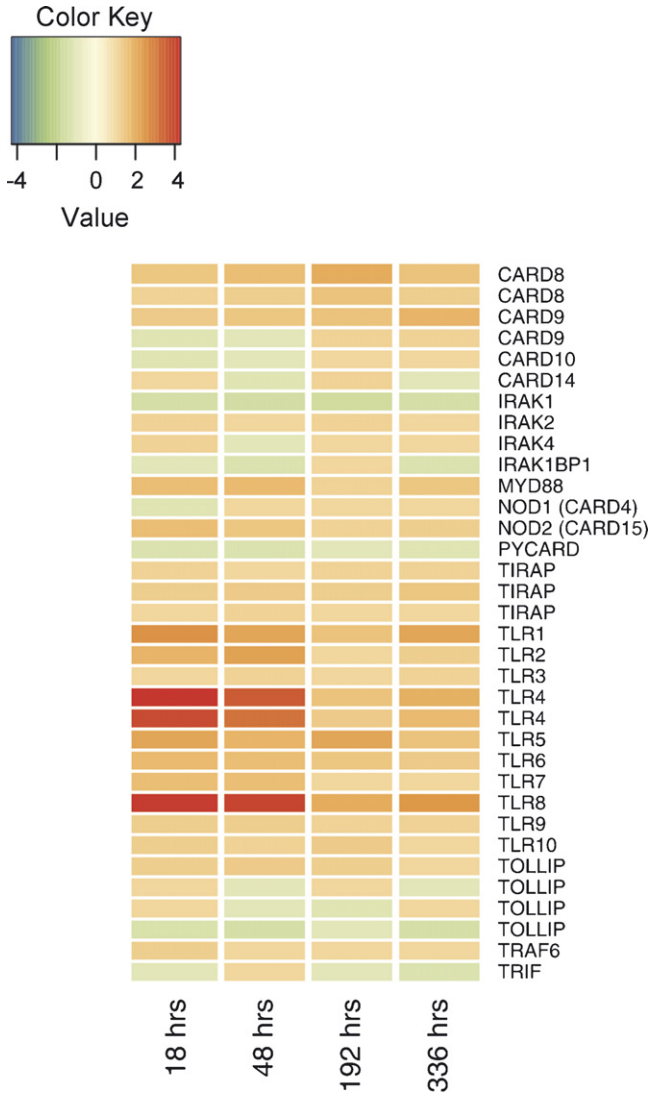

Fig. 7. Heatmap visualization reveals selective up-regulation of TLR/NOD-associated genes post-vaccination. The heatmap represents mean fold-change differences in intensity as compared to the pre-vaccination group for five donors at four post-vaccination time points for the indicated TLR/NOD-associated genes. Multiple listings of a single gene indicate that gene is represented by more than one gene fragment in the array.

time points examined in all volunteers, with moderately higher expression at 18–48 h post-vaccination (Fig. 6). Several downstream mediators of TLR signaling such as *IRAK3*, *CARD8*, *MYD88*, and *TIRAP* were activated at time points immediately post-vaccination (18–48 h) or throughout the course of the study (Fig. 6 and Table 5). Thus, human data as well as murine data suggest at least one possible mechanism by which a large number of innate immune system effectors may be activated in response to LVS vaccination (Katz et al., 2006).

4. Discussion

As modern clinical immunology and molecular biology begin to coalesce in the laboratory, they have allowed careful elucidation of previously anecdotal evidence of host-pathogen interactions. Here, we present an application of transcriptome analysis to the human response to the LVS vaccine. Using highly vigorous controls and statistical methods, we present a gene-level view of host-pathogen interactions that may be predictive of the formation of long-lasting, protective immune responses.

The primary obstacle to transcriptome analysis of vaccine response in humans is compensating for human-to-human variability. The wide range of results from anti-*F. tularensis* microagglutination titers, the current clinical standard for assessment of LVS response, underscore the variability that is expected in human studies (Table 1). Thus, in the search for early predictors of vaccine outcomes, any new standard must endure an enormous range of human genetic diversity and remain consistent. Surprisingly, all statistical analysis of this study showed the LVS vaccine was able to elicit an extremely consistent response (correlation > 0.88) across all genes, at all timepoints, in all volunteers (Figs. 2 and 3A). In addressing the need for universal biomarkers predictive of vaccine outcome, these results are more than reassuring.

It must further be noted that while we sought to examine changes in gene regulation post-vaccination, it is more than reasonable to assume that expression of genes that may be instrumental to vaccine efficacy were not changed in a two-fold-manner and therefore not considered here. Second, our analysis of PBMCs can only be thought of as a surrogate for measuring immune responses taking place in the skin, at the site of vaccination.

While prior reports suggested that human LVS responses are not measurable until day ten post-challenge, largely relied on use of *in vitro* re-stimulation assays to reach their conclusions (Karttunen et al., 1991). Since then, a more clear understanding of the importance of innate responses to the development of adaptive immune response has emerged (Elkins et al., 2003). It follows that the most effective vaccines elicit strong innate immune responses that aid in the development of potent adaptive recall responses. Indeed, as we have reported for humans, and others have shown for mice, early responses within the first 48 h of LVS vaccination influence development of immunity (Telepnev et al., 2005; Isherwood et al., 2005; Fuller et al., 2006; Elkins et al., 2003). Transcriptome analysis distinguishes the first 48 h post vaccination as being the timeframe in which most immune system genes and most genes overall are modulated (Figs. 3B, 4, and 5). The first 48 h are so robust that gene expression at later timepoints (>48 h) is more similar to un-immunized volunteers than to any early point (<48 h) after vaccination (Fig. 3A). It is worthy of note that recent murine transcriptional analyses following aerosol challenge with LVS showed a lack of inflammation-related gene modulation until day 4 (Andersson et al., 2006). Whether or not the mode of infection (intranasal versus intradermal), mechanism of measurement (lung lavage versus PBMC), inoculum (20 CFU versus 1.2×10^9), or obvious differences between human and murine susceptibility to LVS can account for these disparities is unclear. Still, our results clearly have statistically significant responses and uniquely quantify human *in vivo* responses, further encouraging the pursuit of transcriptome analysis as a vigorous alternative to current correlates of immunity for LVS and other vaccines.

At this time, global analysis of gene expression on the scale reported here, is too time-consuming and expensive for the clinical laboratory. Several validated biomarkers would greatly increase the feasibility of transcriptome analysis for vaccine development. Immune-related genes mirrored the overall kinetics of modulated genes following vaccination (Fig. 5). Namely, immune gene regulation was most prevalent at early (<48 h) timepoints. Examination of specific immune genes revealed a patterned response in which innate effector- and pro-inflammatory- related genes dominated early transcripts and were largely upregulated (Tables 2–5). While studies were performed on total PBMC populations, cell-specific activation markers revealed that macrophages, DCs, neutrophils, NK cells, and mast cells were among the cell types that rapidly responded to LVS vaccination by modulating effector-related genes. A similar bias towards innate immune system cells was reported in our previous studies in which human PBMCs were immunophenotyped post-LVS vaccination (Fuller et al., 2006). Recent murine studies in which innate and pro-inflammatory immune responses are demonstrated to be vital to protection against lethal LVS infection are in agreement with the data presented here (Elkins et al., 1992, 2003; Katz et al., 2006; Lindgren et al., 2004, 2005; Mariathasan et al., 2005; Pammit et al., 2006).

Not only are responding genes related in their kinetics, examination of specific markers related to response by effector cells revealed the existence of a concerted anti-LVS response (Figs. 6 and 7). Components of the TLR recognition and signaling pathways were highly represented in kinetic and fold-change analyses (Tables 2–5 and Fig. 7). While murine data also emphasized the importance of TLRs in LVS responses, knockout mouse studies implicate TLR2 as being vital to response (Katz et al., 2006; Malik et al., 2006). In contrast, TLR4 and 8 appeared to be the most upregulated TLRs at early timepoints (<48 h) (Fig. 7). TLR4 is noted for its ability to induce T_H1-biasing, and thus cell-mediated, responses in DCs, lending further support to our hypothesis that cell-mediated, and specifically innate, responses are important to human LVS vaccination (Re and Strominger, 2001; Schnare et al., 2001).

Reports from other groups have implicated the inflammasome pathway as essential to murine response to *F. tularensis*, *s*pecifically IL-1β, IL-18, ASC, and caspase-1 (Mariathasan et al., 2005, 2006). Our data did not reveal any pattern or any statistically significant changes in the aforementioned genes except for IL-18, which did meet cut-off criteria for "up early" (Table 5). Of the caspase genes examined, (CASP1/4/6/8), none made our criteria for being included with the observed kinetic patterns, nor did any show statistically significant changes over the course of the study. Our data involved transcript upregulation but not protein expression, therefore it is difficult to ascertain if differences observed are due to experimental issues (mRNA versus protein), model system (human versus murine), or kinetics (+18, +48, +192, +336 h versus +6 d). Experiments to address these differences are underway in our laboratory.

The pivotal role of DCs in development of acquired immune responses is highlighted by our data. Again, the transcriptome analysis presented herein was performed on total PBMC populations, not isolated cells. However, the strong upregulation of genes related to antigen processing and presentation by professional antigen presenting cells (Fig. 6) along with supportive murine and human studies make inferences regarding DC activation justified (Fuller et al., 2006; Katz et al., 2006). Studies in our laboratory regarding isolated human DC responses to LVS also suggest DC activation and priming of cell mediated immunity does occur *in vitro* (manuscript in preparation).

Transcriptome analysis of human immune events has the potential to revolutionize our understanding of drug development, immunology, and pathogenesis. Results from transcriptome analysis could be used to compare efficacy of different lots of the LVS vaccine and serve as a benchmark with which to compare new anti-*F. tularensis* vaccines. As the need to test anti-*F. tularensis* vaccines in challenge models emerges, transcriptome analysis will allow for a better assessment of how murine or nonhuman primate models compare to human responses to the vaccine. This would allow vaccine evaluation in an animal model that most closely emulates human LVS response and thus better predicts human anti-pathogenic *F. tularensis* strain responses. As with all transcriptional analysis, the correlation with protein expression and/or activity must be addressed. However, it is encouraging to designate a subset of proteins of interest, as well as timepoints in which to examine them. Experiments to examine the correlation between the gene regulation reported here and protein expression and function are currently underway in our laboratory.

Conflict of interest

MWP has financial interest in Gene Logic, Inc., the company that performed the microarray experiments and provided assistance with data analysis for these studies.

Acknowledgements

The authors thank K. Sellers-Meyers for scientific input and E. Torres-Melendez, M.T. Cooper, A.E. Kaczmarek, R. Zamani, K.J. Hachey, L. Anthone, the USAMRIID Special Immunizations Program, and the USAMRIID Clinical Laboratory for excellent technical assistance. The authors also thank L. Ostby of the USAMRIID Visual Information Office for vaccination site photographs. K.C.B. is the recipient of a National Research Council Fellowship. This work was supported by a grant from NIAID-NIH (04-0-HS-002 HHS). The opinions or assertions contained herein are those of the authors and are not to be construed as official policy or as reflecting the views of the Department of the Army or the Department of Defense.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2007.01.037.

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